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Immunopathology and Infectious Diseases

Latent Cytomegalovirus Infection Exacerbates Experimental Colitis

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Inflammatory bowel disease (IBD) severity is positively correlated with cytomegalovirus (CMV) infection. This may reflect CMV triggering and/or exacerbating flares of IBD and/or IBD or immunosuppressive drugs administered to patients with IBD increasing susceptibility to CMV infection. Herein, we performed studies in mice to investigate these possibilities. Mice administered murine CMV (MCMV) developed signs of acute viral infection (malaise and weight loss) and had MCMV loads that were readily detected in numerous organs including the intestine. By 4 weeks, these mice manifested a "latent" infection in which MCMV levels were low but detectable by PCR. Such MCMV infection did not induce acute colitis in either wild-type mice or IL-10$^{-/-}$ mice, which are highly prone to developing colitis. However, underlying MCMV infection in an acute or latent state exacerbated the severity of colitis induced by dextran sodium sulfate (DSS). Such potentiation of DSS colitis by latent MCMV infection seemed to occur without viral reactivation. Whereas initial MCMV infection induced acute alterations in serum and intestinal cytokines, such cytokine levels returned to baseline before administration of DSS. However, the initial infection resulted in lasting elevation of antibodies to commensal bacterial antigens, suggesting that MCMV infection may have potentiated colitis via priming of the intestinal immune response to gut microbiota. Thus, underlying CMV infection can alter mucosal immunity, potentially increasing the tendency of CMV-infected hosts to develop colitis. ***(Am J Pathol 2009, 175:2034–2042; DOI: 10.2353/ajpath.2009.090471)***

Inflammatory bowel disease (IBD) is the collective term for the idiopathic inflammatory diseases of the intestine, whose main forms include Crohn's disease and ulcerative colitis. Ulcerative colitis tends to affect the bowel in a continuous fashion from the rectum, extending a certain distance in the colon and then abruptly stopping. It affects the mucosa and submucosa with ulceration and crypt abscesses. Crohn's disease is a nonspecific granulomatous disease that affects all layers of the bowel wall in a skip-like fashion. Whereas IBD is not generally thought to be caused by a specific pathogen, several lines of evidence indicate an essential role for commensal intestinal bacteria. Specifically, IBD has long been associated with an elevated immune response to the commensal gut microbiota, suggesting that the disease may result from immune dysregulation.[1] More recently, it has been observed that persons with IBD have alterations in both the composition and location of their gut microbiota, suggesting that inability to control the normal commensal microbiota might underlie IBD. However, it is not clear whether these changes play a causative role or, rather, are a consequence of IBD. Analogously, although lack of an association between a specific viral pathogen and IBD argue against a specific viral cause of this disorder, common viruses have been suggested as either modulators or triggers of IBD.[2] However, because patients with IBD are frequently in an immunocompromised state because of poor nutrition and immunosuppressive agents, it has been difficult to decipher the extent to which such common viruses might promote and/or exacerbate IBD.

Human cytomegalovirus (CMV) is a member of the herpes family of viruses. CMV infection is quite common with approximately 40 to 70% of adults being infected in the latent state.[3] Infection is bimodal, occurring through vertical and horizontal transmission in early childhood and again mainly through sexual transmission, in young adults.[4] Latent CMV infection, defined by carriage of the CMV genome without active replication, is commonly

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asymptomatic in immunocompetent individuals whereas active CMV infection is associated with clinical disease such as fever, sore throat and leukopenia. CMV commonly infects fibroblasts, endothelial cells, and myeloid cells.[5] CMV has developed methods for evading host immune system, such as latency and inhibition of apoptosis, and this ability enables reactivation of virus in the immunosuppressed hosts.[6] The prevalence of active CMV infection in the colon is considerably higher in patients with IBD relative to control populations.[7] The majority of such infections are thought to represent reactivation of latent CMV, although there is little formal proof to rule out new infections in this population. In any case, CMV is clearly associated with more severe IBD and thought to be a general detriment to the health of patients with IBD with active CMV infections although the extent to which CMV may activate and/or exacerbate flares of gut inflammation in IBD is not well defined.[8] Moreover, little is known regarding whether latent CMV infection affects either development of IBD and/or may alter severity of disease absentia reactivation. Thus, we used a mouse model of CMV infection to investigate the hypotheses that i) CMV might trigger gut inflammation in a susceptible host and/or ii) that the presence of CMV might alter gut inflammation in response to a well defined trigger of acute colitis, namely the chemical dextran sodium sulfate (DSS). We observed that although murine CMV (MCMV) did not trigger acute inflammation, it promoted mucosal immune responses and increased severity of colitis induced by DSS. Thus, latent CMV infection may alter one's risk for developing intestinal inflammation.

Materials and Methods

Materials

C57BL/6 and IL-10$^{-/-}$ mice, on a C57BL/6 background, were purchased from The Jackson Laboratory (Bar Harbor, ME). All experiments used female mice 4 to 8 weeks of age. MCMV was cultured as described previously.[9] In brief, salivary gland-passed MCMV (Smith strain) was prepared by homogenizing the salivary glands of BALB/c mice infected with 10^4 plaque-forming units (PFU) 3 weeks previously, and aliquots of homogenized supernatants of salivary glands were stored at liquid nitrogen in RPMI 1640 with 5% fetal bovine serum. Flagellin (FliC) was purified from *Salmonella typhimurium,* and purity was verified as described previously.[10] Clostridia-like flagellin (F2) was a gift of Yingzhi Cong and Charles Elson (University of Alabama, Birmingham, AL). Lipopolysaccharide (LPS) was purified from *S. typhimurium*. PCR reagents were purchased from Qiagen (Valencia, CA). PCR primers were purchased from Invitrogen (Carlsbad, CA). DSS was from ICN Biomedicals Inc. (Costa Mesa, CA). All other reagents were purchased from Sigma-Aldrich (St. Louis, MO).

Mouse Treatments

Salivary gland-passaged MCMV (a gift from Prof. Hiroki Yushida, Saga Medical School, Saga, Japan) was suspended in 200 μl of Hanks' balanced salt solution with 3% fetal bovine serum. Mice were infected i.p. with 10^5 PFU (or alternate innoculae as indicated in the text) or as a control were injected with Hanks' balanced salt solution/3% fetal bovine serum. Four weeks later, a subset of MCMV-infected and uninfected mice were given drinking water containing 2.5% dextran sodium sulfate. Body weights were measured at least biweekly thereafter. Mice were euthanized at indicated times, and tissue was harvested and analyzed as described below.

PCR Assessment of Viral Levels

Extraction of MCMV DNA from tissues was performed using a QIAamp DNA Mini Kit (Qiagen). In brief, 25-mg samples of tissue (10 mg of spleen) were sonicated after addition of PBS. A Qiagen lysing buffer and proteinase K were added, and the sample was incubated at 56°C for approximately 3 hours (until completely lysed). RNase A was then added along with Qiagen buffer AL. The sample was incubated at 70°C for 10 minutes and ethanol (96 to 100%) was added. The sample was then centrifuged in a spin column several times using Qiagen buffer AW1 and AW2. The sample was incubated for 5 minutes and centrifuged using distilled water, and the filtrate containing MCMV DNA was collected. Both standard and real-time quantitative PCR were performed using the following primer sequences: 5′-ATCTGGTGCTCCTCAGATCAGCTAA-3′ and 5′-ATTGTTCATTGCCTGGGGAGTTT-3′.

Histopathology

H&E-stained colonic sections were assessed for inflammation by a pathologist (C.O.) blinded to sample origin. For each mouse, three full colonic cross-sections were examined from pieces of tissue that spanned the dist 2 cm of the colon. The scoring system used, based on previous work by us and others,[11,12] was as follows: inflammatory cell scoring (0, rare inflammatory cells in lamina propria; 1, increased granulocytes in the lamina propria; 2, confluence of inflammatory cells extending into the submucosa; 3, transmural extension of the infiltrate) and crypt damage scoring (0, intact crypt; 1, loss of basal one-third; 2, loss of basal two-thirds; 3, entire crypt loss; 4, erosion; 5, confluent erosion). In addition, for each section, the relative proportion of inflammatory cells that were polymorphonuclear (ie, neutrophils) versus mononuclear (mix of plasma cells, monocytes, and lymphocytes) was assessed.

Tissue Myeloperoxidase Activity

Infiltration of neutrophils in colonic tissue was measured using myeloperoxidase enzyme activity, which is a marker for neutrophils by following the standard procedure as described. In brief, the colon samples were

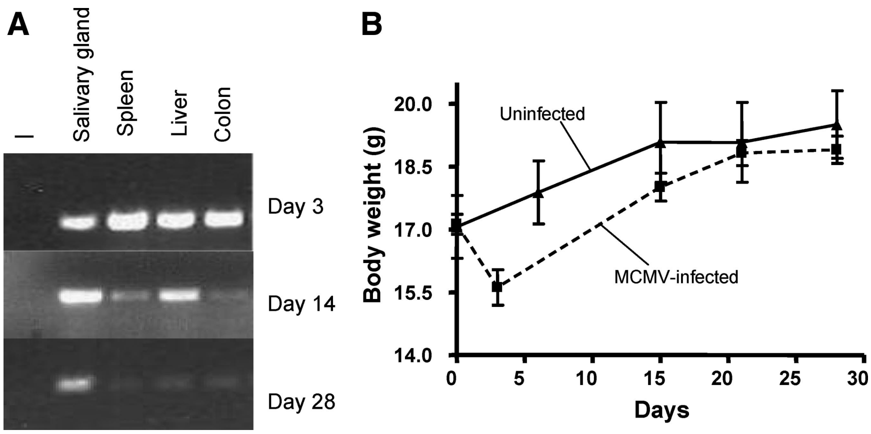

Figure 1. MCMV infected various tissues but did not cause colitis in C57BL/6 mice. C57BL/6 mice were administered 10^5 PFU of MCMV via i.p. injection. **A:** Viral loads were assessed in various tissues by PCR. Data for each time point are from a single mouse. Five mice were analyzed at each time point and showed similar results. The negative control (−) used liver extract from an untreated mouse. **B:** Body weight was monitored biweekly. Results are the mean ± SEM of a single experiment ($n = 5$ mice per condition) and reflective of two experiments that showed similar results. Differences in weight were not statistically significant (NS) by 15 days after infection.

washed thoroughly after which 50 mg/ml tissue was sonicated in 0.5% hexadecyl trimethyl ammonium bromide (in 50 mmol/L phosphate buffer, pH 6.0) and freeze-thawed three times. Samples were then centrifuged. Myeloperoxidase was assayed in the supernatant by adding 1 mg/ml dianisidine dihydrochloride and 5×10^{-4}% H_2O_2, and the change in optical density was measured at 450 nm.

Cytokine Measurements

Sera and intestinal supernatants were evaluated for cytokines using a custom multiplex cytokine enzyme-linked immunosorbent assay kit according to the manufacturer's specifications (Bio-Rad Laboratories, Hercules, CA). Serum was collected in serum separator tubes (BD Microtainer, BD, Franklin Lakes, NJ). Intestinal supernatant samples were collected using 1-cm strips of colon that were washed in Hanks' balanced salt solution and then incubated in 0.5 ml of Dulbecco's modified Eagle's medium for 24 hours. The supernatant was stored at −20°C until analyzed. Analysis was performed by a core facility at Baylor University using a Luminex 100 machine running Bioplex Manager version 4.0 (Bio-Rad Laboratories).

In Vivo *Epithelial Barrier Permeability*

Fluorescein isothiocyanate (FITC)-labeled dextran was used to assess barrier function *in vivo* as described previously.[13] In brief, MCMV-infected or sham-treated 11-week-old C57BL/6 mice were without food and water for 3 hours and then were gavaged with 4 kDa FITC-labeled dextran (0.6 mg/g b.wt.) or horseradish peroxidase (0.02 mg/g b.wt., Sigma-Aldrich). Blood was collected retro-orbitally after 3 hours, and serum was extracted. The fluorescence intensity of each serum sample was measured, and FITC-dextran concentrations were obtained from standard curves generated by serial dilution of FITC-dextran. Horseradish peroxidase was measured using 3,3′,5,5′-tetramethylbenzidine and measuring absorbance at 650 nm.

Measure of Antibodies to Bacterial Products

Sera were isolated from 11-week-old mice that had been infected with MCMV or injected with vehicle 4 weeks previously. Levels of antibodies in sera that recognized flagellin or LPS were measured as described previously.[14,15] In brief, flagellin (FliC, F2) and LPS were applied (100 ng/well for FliC and F2 and 1 μg/well to 96-well plates at 1:100, 1:500, and 1:2500 dilutions). After incubation, the wells were coated with diluted (1:100) sample serum from C57BL/6 mice. After incubation and washing, secondary antibodies and mouse IgG and IgA (GE Healthcare, Little Chalfont, Buckinghamshire, UK) were added to each well at a 1:1000 dilution. Plates were subsequently probed with horseradish peroxidase anti-mouse and exposed to horseradish peroxidase substrate 3,3′,5,5′-tetramethylbenzidine.

Statistics

Statistical analysis was performed with GraphPad Prism software using Student's two-tailed *t*-test. $P < 0.05$ was considered statistically significant.

Results

CMV Colonized Intestine but Did Not Induce Intestinal Inflammation

We hypothesized that CMV might trigger transient intestinal inflammation in wild-type hosts and/or perhaps promote gut inflammation in mice predisposed to developing this disorder. To examine this possibility, we first infected wild-type C57BL/6 mice i.p. with 1×10^5 PFU of MCMV. Such treatment is known to result in acute infection of multiple tissues with peak levels of virus by 3 days postinfection and, a few weeks later, in a chronic low-level infection, analogous to latent CMV infection in humans.[16] In accordance, we observed relatively high levels of MCMV 3 days postinfection that diminished markedly by 21 days but could still be detected by PCR (Figure 1A). MCMV preferentially infects the liver and salivary gland.

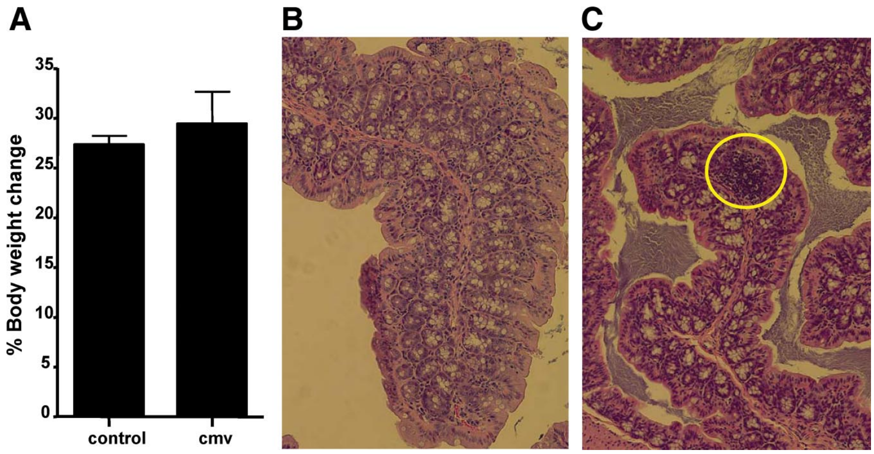

Figure 2. CMV did not trigger acute colitis in IL-10-deficient mice. IL-$10^{-/-}$ mice, on a C57BL/6 background, were injected i.p. with vehicle (Hanks' balanced salt solution) or 10^4 PFU of MCMV. **A:** Weight was monitored for 4 weeks. Results are the mean ± SEM of a single experiment (n = 6 mice per condition) and reflective of two experiments that gave similar results. **B** and **C:** Representative H&E sections of colon from mice administered vehicle (**B**) or MCMV (**C**) 4 weeks after administration. Subepithelial lymphoid aggregates similar to that shown in the **circle** in **B** were observed in four of six MCMV-treated mice and one of six vehicle-treated mice (NS).

Thus, as expected, the level of MCMV was much greater in the liver than in the intestine at all times examined. In accordance with this infection time course/tissue tropism, viral inclusions were occasionally visible in the liver but only during acute infection (data not shown). Relative to control mice, mice infected with MCMV showed typical signs of acute CMV infection such as lethargy and acute weight loss, with a maximum weight loss at 3 days averaging 1.75 g (Figure 1B). Thereafter, infected mice quickly regained a healthy appearance and began to recover the lost weight so that by day 15, the infected mice had body weights that were not significantly different from those of uninfected littermates. Whether taken at the peak of infection (3 days) or at a time when CMV infection is considered latent (28 days), histopathological analysis could not distinguish colons of infected and uninfected wild-type mice. Specifically, we did not observe infiltrates of inflammatory cells, edema, or altered tissue architecture in any of the 10 MCMV-infected mice examined, nor were viral inclusions seen in these intestinal sections. In accordance, at neither time point did CMV infection result in an increase in colonic myeloperoxidase relative to that in uninfected mice (data not shown). Thus, although MCMV can infect the colon of wild-type mice, it does so at low levels and without causing observable inflammation.

We next investigated the extent to which MCMV infection might promote colitis in a host known to be highly susceptible to developing this condition. Specifically, we investigated whether MCMV might trigger colitis in IL-10-deficient mice, which are known to be prone to developing spontaneous robust colitis.[17] In accordance with earlier studies by us and others, IL-$10^{-/-}$ mice on a C57BL/6 background purchased from a commercial source often display only mild lymphocytic colitis. However, we and others have observed that such mice will develop robust colitis if exposed to certain environmental triggers including *Helicobacter* species[18] and the cyclooxygenase inhibitor piroxicam.[19] Moreover, there are presumed to be a variety of other largely uncharacterized potential microbial triggers of colitis in such IL-$10^{-/-}$ mice largely because colitis in these mice is widely observed to be greatly affected by the prevailing microbiota in various mouse facilities. To investigate whether MCMV might be such a trigger, IL-$10^{-/-}$ mice were infected with MCMV as described above. Unexpectedly, this treatment resulted in severe illness and a moribund state in all MCMV-treated mice (n = 6) within 6 days of infection. However, gross and histological analysis of the gut of these mice did not reveal any evidence of inflammation beyond the modest increase in lymphocytes exhibited by uninfected IL-$10^{-/-}$ mice (H&E analysis of three euthanized mice showed an appearance similar to that of uninfected IL-10 knockout mice), nor was there anything remarkable about the spleens in these mice, which often become enlarged in colitic mice. Thus, the unexpected death of IL-$10^{-/-}$ mice after MCMV infection was probably not related to their intestinal phenotype. Given that IL-$10^{-/-}$ mice are known to overproduce proinflammatory cytokines, including tumor necrosis factor-α (TNF-α), interleukin (IL)-12, and interferon-γ (IFN-γ),[20] we speculate that such death reflects an enhanced MCMV-induced cytokine response. In any event, to avoid mortality early in infection so that we could study colitis, we reduced the MCMV inocula to 1×10^4 PFU/mouse, which still led to readily detectable levels of virus in various tissues (by PCR, not shown) but did not cause mortality during the subsequent 28 days that the mice were monitored. Relative to uninfected IL-$10^{-/-}$ mice, the MCMV-infected mice did not show clinical indicators of colitis such as reduced weight gain (Figure 2A) nor the presence of occult blood in stools (gross and occult bleeding were undetectable in either control and MCMV-infected IL-$10^{-/-}$ mice). Histopathological analysis, performed 28 days postinfection, revealed sporadic focal areas of increased lamina propria lymphocytic infiltrates but did not reveal histological evidence of acute inflammation such as inflammatory cells or edema (Figure 2, B and C). Thus, whereas MCMV infection caused modest perturbations in the gut mucosal immune system, it was not sufficient to trigger robust inflammation in these colitis-prone mice.

Underlying CMV Infection Results in More Severe Colitis on Exposure to DSS

Approximately 40 to 70% of human adults are infected with CMV.[3] We hypothesize that such carriage of CMV

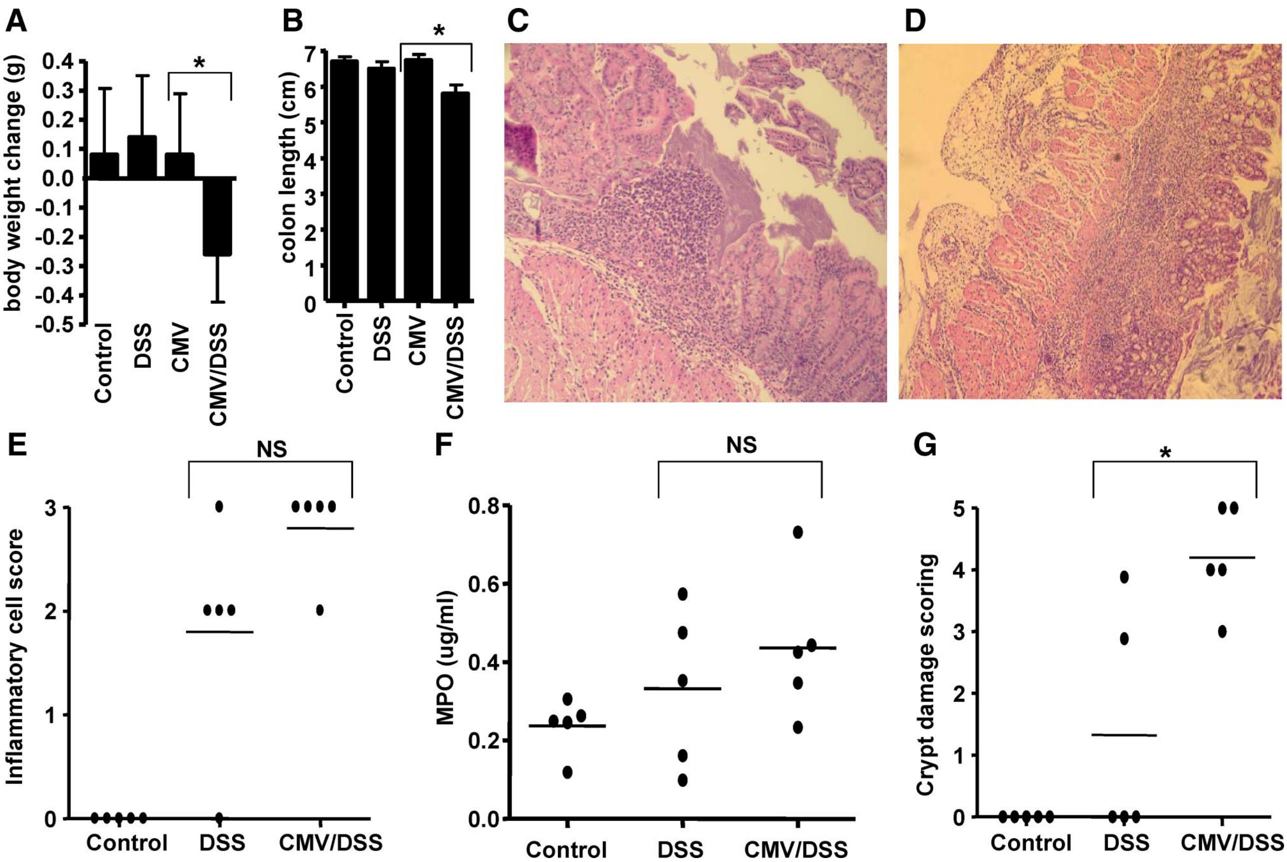

Figure 3. The presence of chronic CMV infection results in exacerbated colitis in response to DSS. C57BL/6 mice were administered vehicle or 10^5 PFU of MCMV via i.p. injection, and 30 days later, such control and chronically MCMV-infected mice were exposed to DSS in drinking water for 7 days, at which time mice were euthanized. Colitis was assessed via changes in weight (**A**), colon length (**B**), histopathological analysis (**C, D, E,** and **G**) and myeloperoxidase (MPO) assay (**F**). Results are from a single experiment (n = 5). Each point in **E–G** represents a result from an individual mouse. This experiment was repeated three times, all of which showed consistently showed significantly increased severity of colitis in MCMV-treated mice. $^*P < 0.05$.

and probably other viral infections might alter one's response to potential triggers of colitis and, hence, alter susceptibility to developing IBD. To begin to test this notion in a well defined animal model, we infected mice with MCMV and, subsequently, exposed them to the chemical colitogen DSS. DSS causes epithelial injury that results in an acute colitis somewhat reminiscent of acute flares of colitis in patients with IBD. C57BL/6 mice were infected with MCMV as described above (or injected with buffer as a control) for 28 days (chronic/latent infection) and then given drinking water containing 2.5% DSS. In mice not infected with MCMV, this moderate concentration of DSS did not result in changes in body weight relative to that of uninfected mice receiving regular drinking water. In contrast, mice chronically infected with MCMV exhibited modest, but statistically significant, weight loss during the course of the 7 days of DSS treatment (Figure 3A). Moreover, in MCMV-infected mice, DSS treatment led to a significant shortening of the colon, a typical feature of robust colitis, which was not present in uninfected mice treated with DSS (Figure 3B). In accordance with these clinical and gross observations, histopathological analysis indicated that mice infected with MCMV had more severe DSS-induced colitis than mice treated with DSS only (Figure 3, C–G). Specifically, the combination of MCMV and DSS moderately elevated levels of inflammatory cells such that most (80%) MCMV/DSS-treated mice showed transmural inflammation whereas the inflammatory infiltrate in mice given DSS only generally did not extend beyond the lamina propria. In general, the more severe inflammatory infiltrates were judged to be an approximately equal mix of neutrophils and mononuclear cells, whereas the milder inflammatory infiltrates were judged to be predominantly (approximately 80%) neutrophilic. The higher levels of inflammatory infiltrates were accompanied by concomitant elevation in colonic levels of myeloperoxidase although this difference did not achieve statistical significance (P = 0.57). However, the most striking difference between these groups of mice was the degree of crypt damage in mice given MCMV/DSS. Specifically, such mice had a much greater degree of crypt loss, erosion, and confluent erosion compared with those of mice given DSS alone.

In response to many challenges, recruitment of inflammatory cells is a healthy aspect of the innate immune response and allows for rapid resolution of inflammation. Thus, we next considered the possibility that the modestly greater level of inflammatory cells in MCMV-infected mice after 7 days of DSS treatment might reflect the possibility that these mice were actually resolving DSS colitis more quickly than un-infected mice. To examine this possibility, mice were administered MCMV and/or

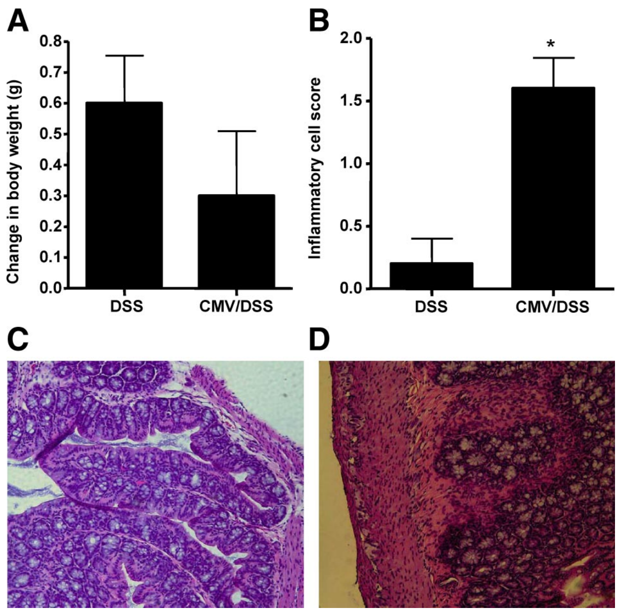

Figure 4. Underlying CMV infection delays recovery from DSS colitis. Mice were treated exactly as described in the legend to Figure 3 except that after 7 days of exposure to DSS, they were returned to regular water and monitored for 7 days before being euthanized. **A:** Change in weight during the 7-day recovery period. **B:** Histopathological scoring of inflammatory cells. **C** and **D:** Representative histological appearance of mice given DSS only (**C**) or DSS/CMV (**D**). Results are from a single experiment that used six mice per condition. $^*P < 0.05$.

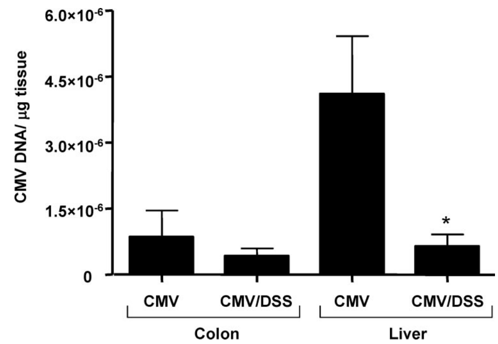

Figure 5. DSS-induced inflammation did not result in CMV reactivation. C57BL/6 mice were administered vehicle or 10^5 PFU of MCMV via i.p. injection, and 30 days later, such control and chronically MCMV-infected mice were exposed to DSS in drinking water for 3 days, at which time they were euthanized and MCMV levels were analyzed by real-time PCR. Results are from a single experiment using six mice per condition. $^*P < 0.05$.

DSS as described above except that, after 7 days of DSS treatment, mice were returned to regular water for and monitored for 7 days. During the recovery period, mice that had been infected with MCMV showed a trend of reduced weight gain relative to mice that had received DSS alone (Figure 4, A–D). By this time both groups of mice lacked detectable damage to their crypt epithelium. However, mice that had been administered MCMV/DSS still exhibited a significant presence of inflammatory cells, predominantly neutrophils, primarily in the lamina propria and, in some cases, a confluence of cells extending into the submucosa, which was not seen in any of the six mice treated with only DSS. Thus, the greater inflammatory pathology seen in MCMV-infected mice at the peak of DSS colitis correlated with a decreased resolution of the inflammation 7 days later.

CMV Infection Promotes Immune Response to Basal Microbiota

We considered several potential mechanisms by which chronic MCMV infection might be increasing the severity of DSS colitis. First, because inflammation in general and cytokines associated with inflammation in particular are known to cause reactivation of some latent viral infections, we first considered the possibility that DSS might trigger MCMV reactivation, ie, lead to an increase in viral load in infected tissues, and the severe colitis might be a reflection of acute MCMV infection combined with DSS-induced inflammation. Indeed, this possibility is consistent by our observation that administering DSS to mice acutely infected with MCMV (3 days) also results in quite severe colitis (data not shown). However, quantitative PCR revealed that DSS did not significantly alter MCMV levels in the colon and actually resulted in reduced MCMV levels in the liver (Figure 5). Thus, the severe colitis induced by DSS in mice chronically infected with MCMV probably did not reflect MCMV reactivation.

It has recently been observed that mice chronically infected with MCMV or gammaherpes virus 68, which also establishes a latent infection in mice, are resistant to oral infection by *Listeria monocytogenes*.[21] In the case of gammaherpes virus 68, such resistance was attributable to chronically elevated serum levels of the cytokines TNF-α and IFN-γ. Thus, we examined whether MCMV infection might affect levels of these and other cytokines in either the serum or intestine. Specifically, we measured levels of KC, IL-6, IL-17, IL-1β, TNF-α, and IFN-γ in the serum and colonic supernatants of control mice and mice acutely (3 days) or chronically (28 days) infected with MCMV (Figure 6, A and B). Whereas acute MCMV infection markedly induced all of these cytokines in the serum (except IL-1β, which was not detected in sera) and elevated KC, IL-6, and TNFα in the intestine (IL-1β, IFNγ, and IL-17 were not detected in intestinal supernatants), we did not observe any significant differences in these parameters in mice chronically infected with MCMV relative to uninfected mice. Thus, our data did not support the notion that chronic activation of innate immunity underlies the severe colitis induced by DSS in MCMV-infected mice.

We next considered the possibility that MCMV might be potentiating colitis via altering mucosal permeability. This hypothesis is based on the evidence in humans suggesting that increases in gut permeability precede robust inflammation[22] in IBD and recent observations in mice that a moderate increase in epithelial permeability predisposes to, but does not itself induce, acute colitis.[23] Thus, mice chronically infected with MCMV and control mice were orally gavaged with FITC-dextran, and the level of FITC-dextran was measured in serum 4 hours later. This approach did not reveal any difference in gut permeability between our test groups of mice (data not shown). Because this approach is not generally viewed as being very sensitive and cannot measure whether

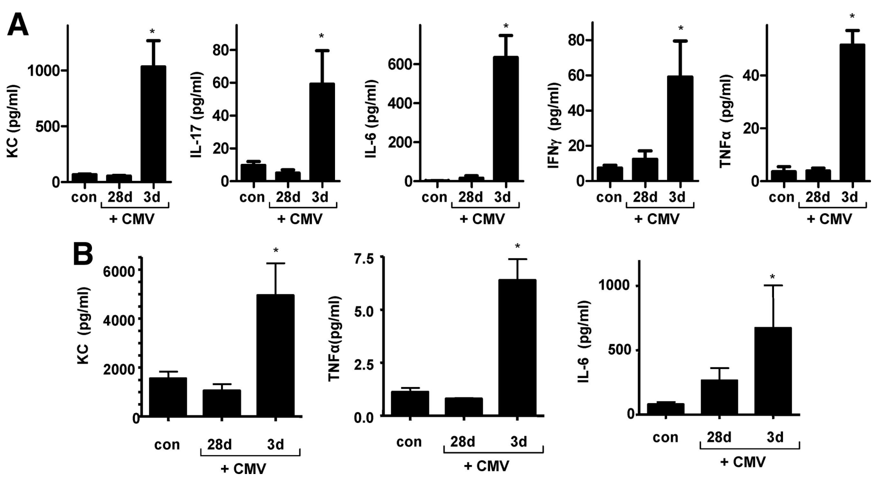

Figure 6. CMV infection induces acute but not prolonged alterations in cytokine secretion. C57BL/6 mice ($n = 6$) were administered vehicle or 10^5 PFU MCMV via i.p. injection. (**A**) Serum and (**B**) intestinal supernatants were isolated on indicated day 28 (day 3 for vehicle-treated mice). Samples were subjected to bead-based cytokine analysis for levels of IFN-γ, IL-1β, IL-6, IL-17, KC, and TNF-α. Significant levels of IL-1β were not detected in the sera of these groups of mice. Significant levels of IL-1β, IL-17, and IFN-γ, were not detected in intestinal supernatants under these conditions. Results are mean ± SEM. *$P < 0.05$ from control (vehicle-treated) mice.

there had been transient changes in gut permeability, we also used another readout of gut permeability. Specifically, as an indirect readout of gut permeability, we measured serum antibodies to the common bacterial components flagellin and LPS, which, as we have shown in mice and humans, probably reflect levels of gut permeability over an extended period.[14,24] In the case of flagellin, we used a typical Gram-negative flagellin, FliC, isolated from *Escherichia coli* and a flagellin cloned out of the spontaneously colitic C3Hejbir mouse strain.[25] In addition, we measured serum antibody responses to *E. coli* LPS. We observed that mice that had been infected with MCMV 4 weeks previously had markedly higher levels of anti-flagellin and anti-LPS antibodies than control mice (Figure 7, A–C). This observation suggests that MCMV infection may make mice more susceptible to a potential trigger of colitis via transiently altering gut mucosal permeability that may prime mucosal immune responses.

Discussion

It is widely recognized that CMV infection of the intestine is associated with severe IBD. It is less clear whether CMV plays a role in complicating and/or triggering flares of disease or, alternatively, is merely an "innocent bystander" that happens to thrive in conditions such as inflammation that characterize active IBD itself and/or the immunosuppressive state that results from agents used to treat this disorder. It has been observed that onset of IBD is associated with CMV infection, suggesting the possibility that CMV infection triggers the onset of IBD in susceptible hosts.[26] However, other studies indicate that the presence of CMV infection in IBD is more common among patients with long-standing disease, arguing against this notion.[27] Thus, we sought to use a well defined mouse model to investigate whether CMV might trigger acute colitis in IL-10-deficient mice, which are which are known to be highly susceptible to developing "spontaneous" colitis. Although IL-10-deficient mice were markedly sensitive to MCMV-induced mortality, we did not observe this virus to induce acute inflammation nor clinically perceptible evidence of intestinal disease in these mice. Thus, our results did not support our hypothesis that CMV had the potential to trigger colitis although this, of course, does not preclude the possibility that CMV might trigger colitis in other strains of mice (or humans) and/or in other environmental conditions.

Another potential explanation for the association between CMV infection and IBD is that CMV infection, although not sufficient to cause colitis, may increase one's susceptibility to developing colitis possibly by potentiating the response to potential triggers of acute colitis. That MCMV infection led to sporadic lamina propria lymphocytic infiltrates in IL-10-deficient mice supports the notion that chronic infection with this virus does indeed modulate mucosal immunity in that mucosal lymphocytes are known to be essential for the eventual development of acute colitis in IL-10-deficient mice. Most importantly, we observed that, in wild-type mice, underlying infection with MCMV markedly potentiated the severity of and duration of the acute colitis that ensues when mice are given DSS, which causes colitis via injuring the gut epithelium. These

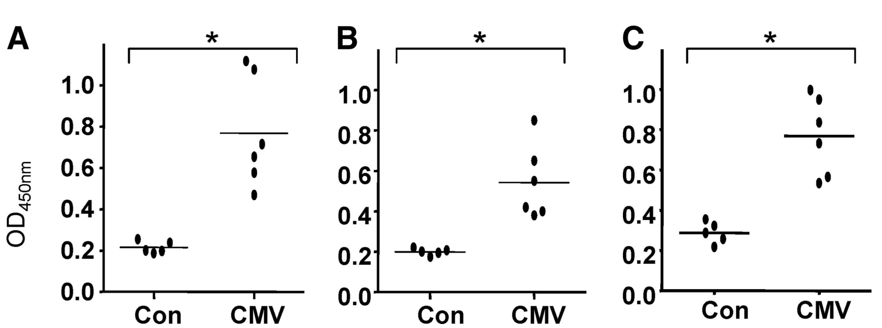

Figure 7. CMV causes an increase in immune response to commensal bacterial antigens. C57BL/6 mice were administered vehicle or 10^5 PFU MCMV via i.p. injection. Serum was isolated 28 days later. Levels of antibodies to common bacterial products were measured via enzyme-linked immunosorbent assay at a serum dilution of 1:1000. **A:** *E. coli* flagellin (FliC). **B:** *Clostridium coccoides* flagellin (F2). **C:** *E. coli* LPS. These results are from a single experiment ($n = 5$) and are representative of two experiments that gave similar results. Each point represents results from an individual mouse. *$P < 0.05$ from control (vehicle-treated) mice.

results support the notion that preexisting CMV infection may make one more prone to exaggerated inflammatory responses and thus may make one more likely to develop IBD. In considering the extent to which this finding is likely to translate to humans, it should be noted that the majority of human CMV infections, in healthy hosts, are considered latent. Likewise, evidence suggests that most MCMV infections in healthy mice also lead to latency,[28] although the notion that MCMV can result in low-level chronic or persistent infections cannot be ruled out. Thus, given that a large percentage of adults are latently infected with CMV, if even a portion of the effect we observed in our mouse model extends to humans, this virus could prove to be playing a role in many cases of IBD. Importantly, that MCMV potentiated the severity of DSS colitis without reactivation suggests that CMV may also be promoting development/severity of IBD even in cases in which CMV would not be detectable by typically used diagnostic assays (ie, by histology).

Whereas the precise molecular mechanism by which underlying MCMV infection potentiated the response to DSS is not yet defined, our observations suggest a key role for MCMV in priming the mucosal immune response. Specifically, we observed that MCMV increased levels of intestinal lymphocytes in IL-10$^{-/-}$ mice and increased antibody responses to commensal microbial antigens in wild-type mice. In both mice and humans, both the presence of commensal bacterial products and, concomitantly, levels of anticommensal bacterial antibodies are known to positively correlate with gut permeability. Moreover, as some persistent viral infections, notably HIV, result in increased intestinal permeability,[29] it seems likely that the elevated levels of anticommensal antibodies observed here reflect the fact that MCMV increased gut permeability, at least transiently. In accordance, moderate increases in gut permeability do not themselves induce colitis but make mice more susceptible to subsequent triggers of colitis.[23] It is possible that MCMV-induced increases in gut permeability result in increased antigen-specific responses that exacerbate the innate immune inflammatory response that ensues in response to the loss of epithelium caused by DSS treatment. In accordance with this possibility we note that, although DSS colitis does not absolutely require adaptive immunity, its severity is modulated by T cells.[30] On the other hand, and perhaps more likely, MCMV-induced increases in gut permeability may simply generally affect the status of the innate immune system hence directly modulating the severity of the acute inflammatory response to DSS. In any event, defining how MCMV infection may alter gut permeability and consequently alter immune responses that mediate colitis will be important to understand both viral pathogenesis and chronic inflammation in the gut.

In conclusion, our results suggest that latent infection by CMV and perhaps other common viruses may modulate mucosal immunity and, consequently, alter one's susceptibility to developing severe acute colitis in response to various challenges and, consequently, may predispose to developing IBD. Such predisposition may occur in the absence of a reactivated viral infection. Should retrospective serological analysis confirm the notion that latent CMV infection increases risk of developing IBD, it may be advisable to consider vaccinating healthy young populations against this virus even in the absence of any risk factors that such individuals are ever likely to be in an immunocompromised state.

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References

1. Strober W, Fuss I, Mannon P: The fundamental basis of inflammatory bowel disease. J Clin Invest 2007, 117:514–521
2. Irving PM, Gibson PR: Infections and IBD. Nat Clin Pract Gastroenterol Hepatol 2008, 5:18–27
3. Roback JD, Hillyer CD, Drew WL, Laycock ME, Luka J, Mocarski ES, Slobedman B, Smith JW, Soderberg-Naucler C, Todd DS, Woxenius S, Busch MP: Multicenter evaluation of PCR methods for detecting CMV DNA in blood donors. Transfusion 2001, 41:1249–1257
4. Mosca F, Pugni L: Cytomegalovirus infection: the state of the art. J Chemother 2007, 19(Suppl 2):46–48
5. Egli A, Binggeli S, Bodaghi S, Dumoulin A, Funk GA, Khanna N, Leuenberger D, Gosert R, Hirsch HH: Cytomegalovirus and polyomavirus BK posttransplant. Nephrol Dial Transplant 2007, 22(Suppl 8):viii72–viii82
6. Limaye AP, Kirby KA, Rubenfeld GD, Leisenring WM, Bulger EM, Neff MJ, Gibran NS, Huang ML, Santo Hayes TK, Corey L, Boeckh M: Cytomegalovirus reactivation in critically ill immunocompetent patients. JAMA 2008, 300:413–422
7. Dimitroulia E, Spanakis N, Konstantinidou AE, Legakis NJ, Tsakris A: Frequent detection of cytomegalovirus in the intestine of patients with inflammatory bowel disease. Inflamm Bowel Dis 2006, 12:879–884
8. Criscuoli V, Rizzuto MR, Cottone M: Cytomegalovirus and inflammatory bowel disease: is there a link? World J Gastroenterol 2006, 12:4813–4818
9. Roback JD, Hossain MS, Lezhava L, Gorechlad JW, Alexander SA, Jaye DL, Mittelstaedt S, Talib S, Hearst JE, Hillyer CD, Waller EK: Allogeneic T cells treated with amotosalen prevent lethal cytomegalovirus disease without producing graft-versus-host disease following bone marrow transplantation. J Immunol 2003, 171:6023–6031
10. Gewirtz AT, Simon PO Jr, Schmitt CK, Taylor LJ, Hagedorn CH, O'Brien AD, Neish AS, Madara JL: Salmonella typhimurium translocates flagellin across intestinal epithelia, inducing a proinflammatory response. J Clin Invest 2001, 107:99–109
11. Katakura K, Lee J, Rachmilewitz D, Li G, Eckmann L, Raz E: Toll-like receptor 9-induced type I IFN protects mice from experimental colitis. J Clin Invest 2005, 115:695–702
12. Vijay-Kumar M, Wu H, Aitken J, Kolachala VL, Neish AS, Sitaraman SV, Gewirtz AT: Activation of Toll-like receptor 3 protects against DSS-induced acute colitis. Inflamm Bowel Dis 2007, 13:856–864
13. Vijay-Kumar M, Sanders CJ, Taylor RT, Kumar A, Aitken JD, Sitaraman SV, Neish AS, Uematsu S, Akira S, Williams IR, Gewirtz AT: Deletion of TLR5 results in spontaneous colitis in mice. J Clin Invest 2007, 117:3909–3921
14. Sanders CJ, Yu Y, Moore DA 3rd, Williams IR, Gewirtz AT: Humoral immune response to flagellin requires T cells and activation of innate immunity. J Immunol 2006, 177:2810–2818
15. Sitaraman SV, Klapproth JM, Moore DA 3rd, Landers C, Targan S, Williams IR, Gewirtz AT: Elevated flagellin-specific immunoglobulins in Crohn's disease. Am J Physiol Gastrointest Liver Physiol 2005, 288:G403–G406
16. Kern ER: Pivotal role of animal models in the development of new therapies for cytomegalovirus infections. Antiviral Res 2006, 71:164–171

17. Kühn R, Lohler J, Rennick D, Rajewsky K, Muller W: Interleukin-10-deficient mice develop chronic enterocolitis. Cell 1993, 75:263–274
18. Kullberg MC, Ward JM, Gorelick PL, Caspar P, Hieny S, Cheever A, Jankovic D, Sher A: Helicobacter hepaticus triggers colitis in specific-pathogen-free interleukin-10 (IL-10)-deficient mice through an IL-12- and gamma-interferon-dependent mechanism. Infect Immun 1998, 66:5157–5166
19. Berg DJ, Zhang J, Weinstock JV, Ismail HF, Earle KA, Alila H, Pamukcu R, Moore S, Lynch RG: Rapid development of colitis in NSAID-treated IL-10-deficient mice. Gastroenterology 2002, 123:1527–1542
20. Gazzinelli RT, Wysocka M, Hieny S, Scharton-Kersten T, Cheever A, Kuhn R, Muller W, Trinchieri G, Sher A: In the absence of endogenous IL-10, mice acutely infected with Toxoplasma gondii succumb to a lethal immune response dependent on $CD4^+$ T cells and accompanied by overproduction of IL-12, IFN-γ and TNF-α. J Immunol 1996, 157:798–805
21. Barton ES, White DW, Cathelyn JS, Brett-McClellan KA, Engle M, Diamond MS, Miller VL, Virgin HW 4th: Herpesvirus latency confers symbiotic protection from bacterial infection. Nature 2007, 447:326–329
22. Hollander D: Intestinal permeability, leaky gut, and intestinal disorders. Curr Gastroenterol Rep 1999, 1:410–416
23. Su L, Shen L, Clayburgh DR, Nalle SC, Sullivan EA, Meddings JB, Abraham C, Turner JR: Targeted epithelial tight junction dysfunction causes immune activation and contributes to development of experimental colitis. Gastroenterology 2009, 136:551–563
24. Ziegler TR, Luo M, Estivariz CF, Moore DA 3rd, Sitaraman SV, Hao L, Bazargan N, Klapproth JM, Tian J, Galloway JR, Leader LM, Jones DP, Gewirtz AT: Detectable serum flagellin and lipopolysaccharide and upregulated anti-flagellin and lipopolysaccharide immunoglobulins in human short bowel syndrome. Am J Physiol Regul Integr Comp Physiol 2008, 294:R402–R410
25. Lodes MJ, Cong Y, Elson CO, Mohamath R, Landers CJ, Targan SR, Fort M, Hershberg RM: Bacterial flagellin is a dominant antigen in Crohn disease. J Clin Invest 2004, 113:1296–1306
26. Orvar K, Murray J, Carmen G, Conklin J: Cytomegalovirus infection associated with onset of inflammatory bowel disease. Dig Dis Sci 1993, 38:2307–2310
27. Pfau P, Kochman ML, Furth EE, Lichtenstein GR: Cytomegalovirus colitis complicating ulcerative colitis in the steroid-naive patient. Am J Gastroenterol 2001, 96:895–899
28. Pollock JL, Virgin HW 4th: Latency, without persistence, of murine cytomegalovirus in the spleen and kidney. J Virol 1995, 69:1762–1768
29. Brenchley JM, Douek DC: The mucosal barrier and immune activation in HIV pathogenesis. Curr Opin HIV AIDS 2008, 3:356–361
30. Kim TW, Seo JN, Suh YH, Park HJ, Kim JH, Kim JY, Oh KI: Involvement of lymphocytes in dextran sulfate sodium-induced experimental colitis. World J Gastroenterol 2006, 12:302–305